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THE *LESSONIA NIGRESCENS* SPECIES COMPLEX (LAMINARIALES, PHAEOPHYCEAE) SHOWS STRICT PARAPATRY AND COMPLETE REPRODUCTIVE ISOLATION IN A SECONDARY CONTACT ZONE[1]

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During secondary contact between phylogenetically closely related species (sibling species) having diverged in allopatry, the maintenance of species integrity depends on intrinsic and extrinsic reproductive barriers. In kelps (Phaeophyceae), the observations of hybrids in laboratory conditions suggest that reproductive isolation is incomplete. However, not all interspecific crosses are successful, and very few hybrids have been observed in nature, despite the co-occurrence of many kelp species in sympatry. This suggests that there are reproductive barriers that maintain species integrity. In this study, we characterized the fine genetic structure of a secondary contact zone to clarify the extent of reproductive isolation between two sister species. In *Lessonia nigrescens* Bory (Laminariales, Phaeophyta) species complex, two cryptic species have been recently found out from gene phylogenies, and—waiting for a formal taxonomic description—we used their geographic distribution to name them (northern and southern species). We studied 12 populations, distributed along 50 km of coastline, and employed two molecular approaches, assigning individuals to phylogenetic species according to a diagnostic mitochondrial marker (351 individuals analyzed) and quantifying interspecific gene flow with four microsatellite markers (248 individuals analyzed). No hybridization or introgression was revealed, indicating complete reproductive isolation in natural conditions. Unexpectedly, our study demonstrated that the two species were strictly segregated in space. This absence of co-occurrence along the contact zone can partially explain the lack of hybridization, raising new interesting questions as to the mechanisms that limit sympatry at small spatial scales.

Key index words: **biological species concept; gene flow; hybridization; kelp; microsatellite; parapatry; reproductive isolation; secondary contact**

Abbreviation: **AMOVA, analysis of molecular variance**

The interactions between local adaptation and dispersal are the main mechanisms behind the evolution of species ranges (Bridle and Vines 2007, Eckert et al. 2008). These mechanisms are particularly important in areas of secondary contact between sister taxa. In these areas, the processes that occur at range margins depend primarily on the degree of reproductive isolation between phylogenetically closely related species (sister species). The absence of reproductive barriers leads to the fusion of taxa into a single species, while partial reproductive isolation can lead to the formation of a hybrid zone whose structure depends on the degree of genetic and ecological differentiation between taxa, their dispersal rate, and the fitness of hybrid offspring (Hewitt 1988, Coyne and Orr 2004, Goldberg and Lande 2007).

Contact zones between sibling species in marine environments have been studied mainly in invertebrate species. As a classic example, the secondary contact between the mussel species *Mytilus galloprovincialis* and *M. edulis* has resulted in a mosaic hybrid zone composed of patches with various introgression rates (Bierne et al. 2003). By using a

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combination of approaches (experimental crosses and population genetics), the maintenance of this mosaic structure in mussels despite their high dispersal capacity is attributed to the combined effects of (i) habitat selection limiting effective dispersal and (ii) genetic incompatibilities that reduce the fitness of hybrids (see Bierne et al. 2003, 2006).

Discrepancies between molecular markers have uncovered cases of putative hybridization between different species (e.g., red algae: Zuccarello et al. 2005, Brodie and Zuccarello 2006, Niwa et al. 2009, Destombe et al. 2010; brown algae: Coyer et al. 2006, 2007, Robba et al. 2006, Lane et al. 2007, Fraser et al. 2009). However, few studies on hybridization have been conducted in natural populations of seaweeds, with the notable exception of the *Fucus* genus (Phaeophyceae). In contrast to mussels, sister *Fucus* species have largely overlapping species ranges but occupy different heights on the shore: they are clearly sympatric on large spatial scales, but parapatric on the scale of a single shore. In *Fucus* species, a combination of cytoplasmic DNA sequences and biparentally inherited markers (microsatellites) has demonstrated that reproductive isolation between these morphological species is incomplete (Wallace et al. 2004, Billard et al. 2005a, Engel et al. 2005, Coyer et al. 2007) and can even lead to adaptation in new habitats (Coyer et al. 2006). These studies in natural populations corroborate results from interspecific crosses that have been performed in controlled conditions (Sauvageau 1909, Stomps 1911, Kniep 1925, see also Coyer et al. 2002, Billard et al. 2005b). The maintenance of a hybrid zone and divergence observed at different sites and in different *Fucus* species are attributed to the combined effects of habitat selection, highly limited gamete dispersal, and evolution of reproductive isolation (Pereyra et al. 2009, Billard et al. 2010).

In kelps, hybrids between congeneric species were also observed under laboratory conditions (*Alaria*: Kraan and Guiry 2000, Kraan et al. 2001; *Laminaria*: Druehl et al. 2005), as well as between genera of the same family (Sanbonsuga and Neushul 1978, Lewis and Neushul 1995, Liptack and Druehl 2000), but rarely in natural populations (but see Coyer and Zaugghaglund 1982, Coyer et al. 1992; see for review Bartsch et al. 2008 and Bolton 2010). Thus, the absence of reproductive barriers in the lab does not demonstrate that hybridization cannot occur in the field. For example, spatial and temporal reproductive isolation can only be encountered in the field. Indeed, the sympatric distribution of many species of Laminariales in some areas of the world (e.g., Canada: Druehl 1970; Brittany: Lüning 1990; Japan: Yoshida et al. 2000) suggests that reproductive barriers generally occur and maintain species integrity. Nevertheless, with the use of molecular tools, the partial discrepancy observed between mitochondrial and nuclear markers could be attributed to incomplete reproductive barriers after secondary contact between divergent lineages of *Alaria* species in natural populations of the North Pacific (Lane et al. 2007). Two hypotheses have been proposed to explain the somewhat incomplete reproductive isolation between related species of Phaeophyceae: (i) recent, rapid radiation of this taxonomic group (de Reviers and Rousseau 1999, Draisma et al. 2001) and (ii) all kelps sharing the same bouquet of pheromones by which the female gamete controls the release and attraction of male gametes (Müller and Maier 1985; see for review Maier and Müller 1986 and Pohnert and Boland 2002).

Within the marine macroalgae, many cryptic species have been discovered using the barcoding technique (e.g., Saunders 2005, Lane et al. 2007, Guillemin et al. 2008). We refer here to cryptic species as ''two or more species [that] are, or have been, classified as a single nominal species because they are at least significantly morphologically indistinguishable'' (Bickford et al. 2006, p. 148). Whether the differentiated lineages of cryptic species fit into the biological species concept partly relies on the existence of reproductive barriers. The discovery of cryptic species has led to a taxonomic reexamination of some taxa (e.g., for review, in red algae: Brodie and Zuccarello 2006; in Laminariales: Bolton 2010). In other cases, cryptic species remain long after their identification without taxonomic characterization and therefore with no corresponding Latin name (e.g., in *Durvillaea antarctica*: Fraser et al. 2009; in *Gracilaria chilensis:* Cohen et al. 2004). Two phylogenetic species have recently been found out within the morphological species *L. nigrescens* (Tellier et al. 2009). This study, using nuclear, mitochondrial, and chloroplast markers revealed two strongly divergent lineages within the taxon *L. nigrescens*, with a divergence of the same order of magnitude than between other related species of the genus *Lessonia* (Tellier et al. 2009). These results suggest that these two lineages correspond to two distinct phylogenetic species. Waiting for a taxonomic revision of the genus *Lessonia* and assignation of new species Latin names, we adopted the following terms to designate the (phylogenetic) cryptic species: the ''northern species'' and the ''southern species'' (following Oppliger et al. 2011). These two cryptic species have contrasting distribution ranges along the Chilean coast: the northern species occurs between 16 and 30° S latitude, and the southern species stretches between 29 and 41° S (Fig. 1). The contact zone located between 29 and 30° S has been described as a mosaic of sites occupied by one species or the other (Tellier et al. 2009), rather than as a true gradual transition zone, with one species replacing the other. Moreover, this area of *L. nigrescens* species' borders corresponds to a biogeographic transition zone that constitutes a range margin for many other coastal invertebrates and macroalgal species (Camus 2001) and a region of important

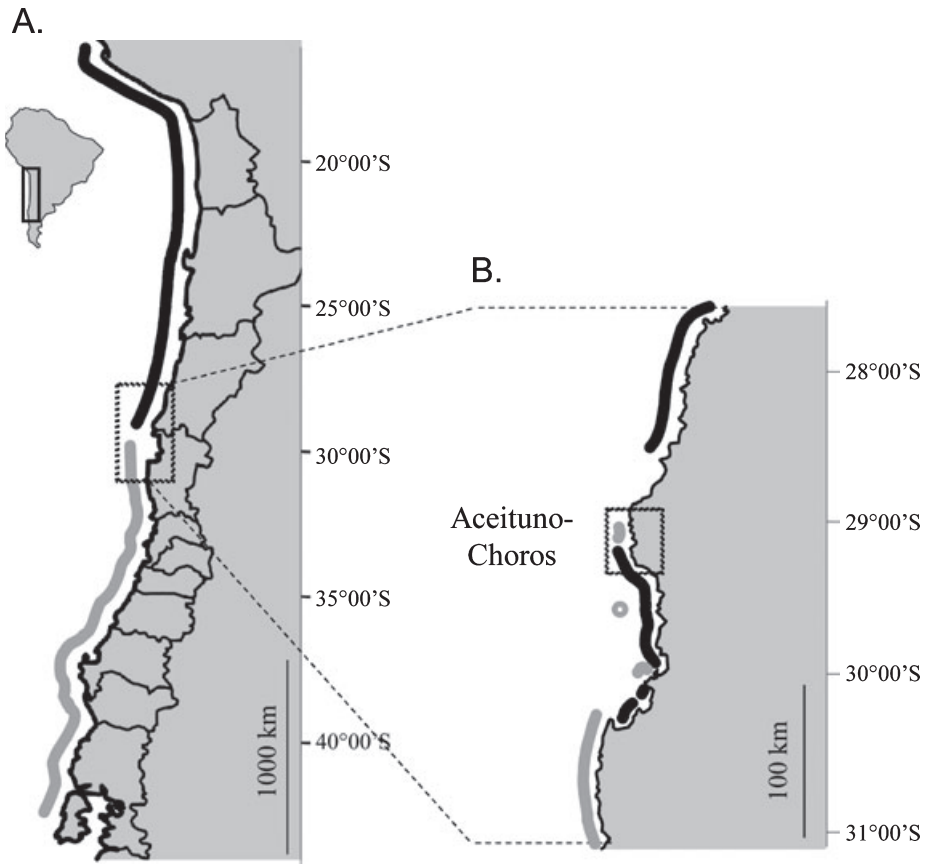

FIG. 1. Geographic distribution of the northern and southern phylogenetic species of *Lessonia nigrescens*, according to Tellier et al. (2009). (A) At the scale of Chilean coasts. (B) At the scale of the transition zone (28°–31° S). Southern species is represented in gray, northern species in black. Species were determined using the mitochondrial marker atp8/trnS.

changes in recruitment pattern of several invertebrate species (Broitman et al. 2001, Navarrete et al. 2005). This concordance between biogeographic patterns and phylogeographic discontinuity is likely to be linked to historical and/or contemporary processes that affect species distributions (Tellier et al. 2009). At a mesoscale, the transition zone is concordant with some present-day coastal oceanographic features, such as strong kinetic eddies ~30° S and a maximum wind stress for upwelling conditions (Hormazabal et al. 2004, Tapia et al. 2009; see for review Thiel et al. 2007). However, the pattern of the transition zone at a smaller spatial scale (continuous gradient vs. mosaic of patches) was not studied by Tellier et al. (2009) and raises the question as to how this contact zone between cryptic species is maintained. Two kinds of contact zones (ecotone vs. tension zones) have been described in the literature to distinguish among the processes acting to retain species integrity (Hewitt 1988, Coyne and Orr 2004, Goldberg and Lande 2007). In ecotone zones, both species are maintained by differential fitness of the parental genotypes along an environmental gradient, whereas in tension zones, species integrity is preserved by an equilibrium between dispersal and selection against hybrids. Theory predicts opposite effects of geographic barriers on the location of the transition zone (Hewitt 1988, Goldberg and Lande 2007): while ecotone zones are repelled by dispersal barriers, tension zones are attracted by habitat discontinuity and thus generally coincide with geographic barriers. The objective of this study was to characterize the distribution pattern of the species in the contact zone and to detect the extent of putative hybridization.

More specifically, we characterized the genetic structure in the contact zone between the two cryptic species to determine (i) the species distribution at small spatial scales and whether the changeover between patches of distinct species is abrupt or gradual, and (ii) the proportion of introgressed and parental genotypes in local populations to ascertain whether there is a hybrid zone and, if so, how populations are distributed spatially. Characterizing genetic structure in the contact zone allowed us to (iii) determine if there is concordance between a dispersal barrier or a habitat discontinuity and the exact spatial location of the contact zone. Using a set of four microsatellite markers and one mitochondrial marker, we determined the patterns of species distribution, the frequency of hybridization, and the rate of introgression.

TABLE 1. Sampled sites in the Aceituno-Choros contact zone. For each site, the abbreviated site name, geographic coordinates, and the number of individuals analyzed (Nmt: mitochondrial marker; Nmsat: microsatellite markers) are given. Dashed lines represent the three habitat discontinuities in the study region, constituted of long stretches of sandy beach: Aceituno beach (between site APN and APS, 5 km), Ermitaño beach (between site ERMS and APON, 1.5 km), and Choros beach (between CHN and CHS, 17 km).

Sampling site	Abbreviation	Latitude	Longitude	Nmt	Nmsat
Cueva del Pirata	CPI	29°01′34″ S	71°29′47″ W	30	23
Ch. Aceituno	ACE	29°03′58″ S	71°29′26″ W	30	13
ACE playa Norte	APN	29°05′08″ S	71°29′46″ W	28	22
ACE playa Sur	APS	29°06′55″ S	71°28′15″ W	30	26
Ermitaño	ERM	29°08′44″ S	71°30′09″ W	30	24
Ermitaño Sur	ERMS	29°10′04″ S	71°29′26″ W	29	22
Apolillado Norte	APON	29°10′51″ S	71°29′29″ W	24	20
Apolillado	APO	29°11′02″ S	71°29′48″ W	30	20
Choros Ventana	CHV	29°12′57″ S	71°28′23″ W	30	18
Choros Barranca	CHB	29°14′28″ S	71°27′52″ W	30	20
Choros Norte	CHN	29°15′26″ S	71°27′09″ W	30	20
Choros Sur	CHS	29°21′10″ S	71°19′47″ W	30	20

MATERIALS AND METHODS

Characteristics of the life cycle of the study species. The life cycle of species in the *Lessonia* genus is characterized by the alternation of a microscopic haploid phase (male and female gametophytes) and a macroscopic diploid phase (sporophyte). Dispersal of male gametes is assumed to be very limited (less than a millimeter, Boland 1995) and is controlled by chemiotaxis, whereby the male gamete is attracted to pheromones produced by the female gamete. The sporophyte germinates directly on the female gametophyte and is not dispersed (Olivari 1974, Avila et al. 1985). The meiospores released by the sporophytes are thus the main dispersal vector, although their life span is generally short (<24 h, Parada 2001).

Sampling. A total of 351 individuals from 12 sites were sampled in the Aceituno-Choros region, which is the northernmost range limit described for the southern species (29°03′ S) (Tellier et al. 2009) (Table 1). This region constitutes a contact zone between the two species, where the southern species forms an enclave within the species range of the northern species (Fig. 1; Tellier et al. 2009). The study zone stretched over a little more than 50 km (Fig. 2). Sites were sampled regularly along the coast, approximately every 5 km. Furthermore, to test for the possible presence of barriers to gene flow, we expressly sampled sites at either end of each of the three longest stretches of sandy beach identified in the study zone, ranging from 1.5 to 17 km (see Fig. 2). At each site, 30 individuals were randomly collected along a 100–150 m transect. Each individual sample was stored in silica gel until DNA extraction.

DNA extraction, molecular species identification, and genotyping. DNA was extracted following the method described in Tellier et al. (2009). The atp8/trnS mitochondrial marker was amplified using PCR conditions as in Tellier et al. (2009), and the two species were distinguished based on a length polymorphism (Tellier et al. 2009). Mitochondrial haplotype sizes were determined by migrating PCR products on a 2% agarose gel. Previously sequenced individuals of each species (Tellier et al. 2009) were used as positive controls in each gel. We estimated that no additional sequencing was necessary, because of the complete congruence between species and the fragment sizes for >1,000 individuals previously analyzed (Tellier et al. 2009).

Four microsatellite markers (LESS1T3, LESS1T9, LESS2D1, and LESS2D22; Faugeron et al. 2009) were used to characterize the genetic structure of both species. PCR conditions were according to Faugeron et al. (2009). PCR products were analyzed on an ABI-PRISM 310 automatic sequencer (Perkin Elmer, Waltham, MA, USA) using the 500 ROX size standard (Applied Biosystems, Foster City, CA, USA). Chromatograms were interpreted using GeneMarker software ver. 1.75 (SoftGenetics, State College, PA, USA) and input files for statistical packages were created with the software package CREATE ver. 1.1 (Coombs et al. 2007).

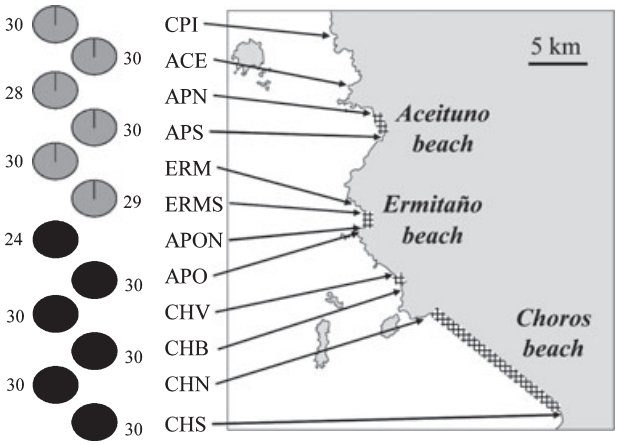

FIG. 2. Geographic distribution of the northern and southern species of *Lessonia nigrescens* in the study region: results from the mitochondrial marker. Individuals were unambiguously assigned to one of the two phylogenetic species. For each site, the number of individuals belonging to the northern species (black) and to the southern species (gray) are given, as well as the number of individuals analyzed at each site. Note: all pie charts are composed of just one color, thereby indicating the absence of species co-occurrence within any single site.

Data analysis. For each species and for each sampling site, we calculated allele frequencies at each locus and the mean number of alleles per locus (N_A) using the GENETIX software package ver. 4.05.2 (Belkhir et al. 1999). To correct N_A values for different sample sizes, we calculated allelic richness (A) by applying the rarefaction method as implemented in the HP-Rare software package ver. June-6-2006 (Kalinowski 2005). Diversity indices (gene diversity He, and allelic richness) for each site were compared between species using Mann–Whitney U-tests implemented in the Minitab software package ver. 13.20 (State College, PA, USA).

Genetic differentiation indices (F_{ST} indices) between pairs of sites and between groups of individuals (where groups were defined by the species to which they were assigned or by sampling site) were calculated using Arlequin ver. 3.1.1 (Excoffier et al. 2005). Significance of F_{ST} values was determined using a nonparametric test based on 1,000 permutations. The threshold value α was set at 0.05, which was subsequently corrected using a Bonferroni correction to account for the high number of tests. Species differentiation was also tested using a hierarchical analysis of molecular variance (AMOVA) by defining a priori species groups based on the mitochondrial marker. In addition, an AMOVA was implemented to quantify the genetic variability explained by habitat discontinuities (i.e., stretches of sandy beach). However, since only one site was sampled to the south of Choros beach, the AMOVA could not be performed for this site. AMOVA analyses were carried out using the Arlequin software package with allele frequency-based statistics (F_{ST} analogs, Excoffier et al. 1992).

RESULTS

Species' geographic distribution. Using the length polymorphism of the mitochondrial marker, each of the 351 sampled individuals was unambiguously assigned to one of the two species. Furthermore, species never co-occurred at the same site (Fig. 2), despite the high number of individuals analyzed at each site (24–30, Table 1). Moreover, species showed a clear geographic separation: sites occupied by the southern species were contiguous and strictly separated from those occupied by the northern species, with Ermitaño sandy beach marking the abrupt species changeover (Fig. 2).

Interspecific genetic structure. A subsample of 248 individuals were genotyped (Table 1), 130 individuals from the southern species and 118 individuals of the northern species, as defined by the mitochondrial marker. The microsatellite genotypes were perfectly congruent with the mitochondrial genotypes: all alleles at all four loci were diagnostic, that is, they were observed exclusively in only one of the two species (Fig. 3). Allele distributions were dissimilar between species, sometimes with great differences in allele size (up to 7 repeat motifs, Fig. 3).

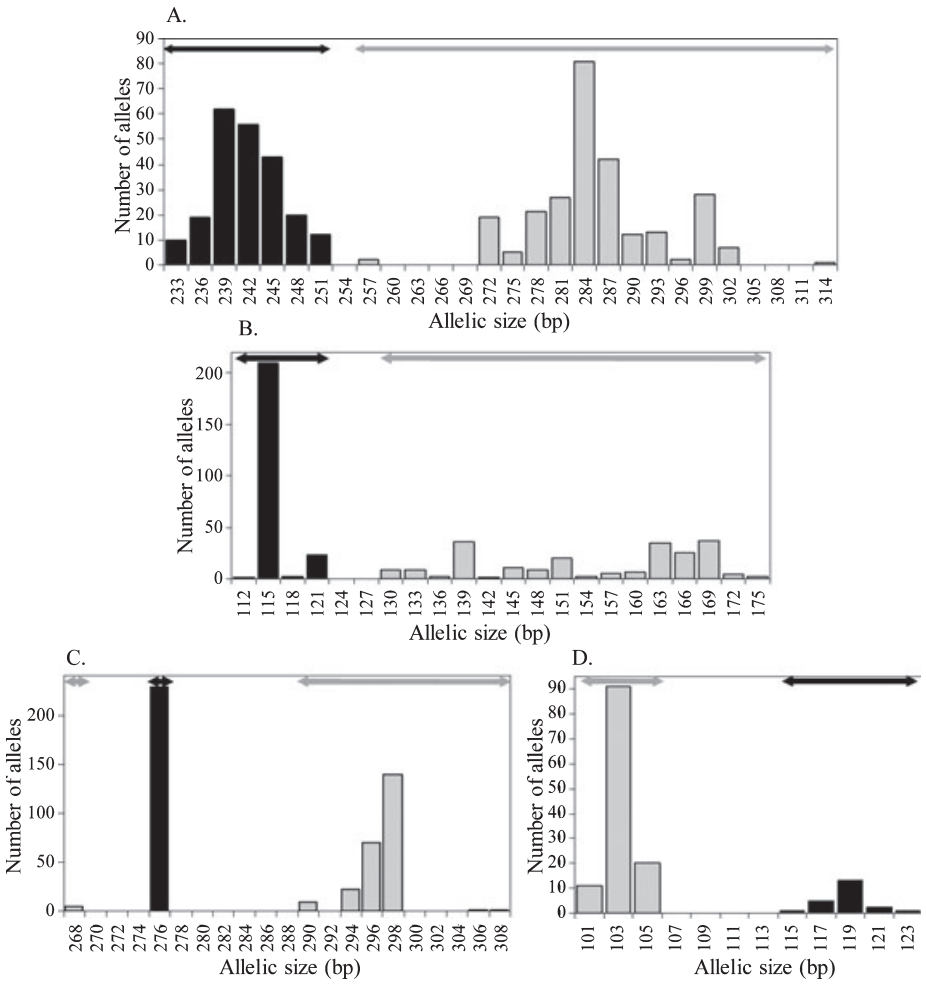

FIG. 3. Allele size distribution for the four microsatellite loci in the study region. Alleles observed in the southern species (as determined with the mitochondrial marker) are given in gray and in the northern species in black. This color code clearly illustrates that each allele was observed only in one species or another, never both. (A) Locus LESS1T9. (B) Locus LESS1T3. (C) Locus LESS2D1. (D) Locus LESS2D2.

For locus LESS1T1, allele distributions of each species overlapped, but none of the alleles were common to both species. Locus LESS2D22 was difficult to amplify and resulted in a high rate of missing data; this locus was therefore excluded from subsequent analyses (Fig. 3).

Pair-wise F_{ST} values were all significantly different from zero for comparisons between sites of different species (Table S1 in the supplementary material), reflecting the absence of shared alleles between species. In contrast, differentiation between sites within a species was generally not significant (Table S1). F_{ST} values between species ranged from 0.355 to 0.561, which are high in comparison to those observed between sites of a given species (from 0 to 0.250).

Diversity indices, calculated using the rarefaction method, showed great differences between species (Table 2): on average, they were significantly lower in populations of the northern species than in populations of the southern species (allele richness, gene diversity, $P < 0.05$, Table 2). The southern species showed an allelic richness of 11.8 alleles per locus compared to 3.9 for the northern species.

Intraspecific genetic structure. The hierarchical analysis AMOVA reconfirmed the significant differentiation between species, which accounted for 45.43% of the total variance (Table 3a). The differentiation between sites within a species, although significant, only explained 2.65% of the total variance. Finally, 51.92% of the variance was attributed to intrasite genetic variation. Within the southern species, the AMOVA showed that the greatest proportion of the variance could be explained by intrasite variation, whereas the tested spatial structure (separation by a stretch of sandy beach) was not significant ($P = 0.10$, Table 3b).

TABLE 2. Diversity indices of populations in the Aceituno-Choros contact zone (microsatellite markers): allelic richness (*A*) and gene diversity (*He*).

Species	Site	Allelic richness	*He*
Southern	CPI	7.02	0.8164
	ACE	6.37	0.7476
	APN	5.25	0.7397
	APS	5.09	0.7072
	ERM	5.77	0.6995
	ERMS	4.64	0.6119
Northern	APON	2.67	0.2771
	APO	2.85	0.3014
	CHV	2.98	0.3740
	CHB	2.94	0.3449
	CHN	2.30	0.2375
	CHS	2.82	0.3101

DISCUSSION

Using a combination of mitochondrial and nuclear markers, we demonstrated (i) a complete geographic segregation of the northern and southern species of *L. nigrescens* and (ii) a concordance between habitat discontinuity (sandy beaches) and the exact spatial location of the contact zone. These two major results are discussed below.

Species' geographic distribution. No sympatric zone was detected in this study. At each and every sampling site, only one of the two species was present. This strict geographic species segregation was even maintained at a very small spatial scale (e.g., across 1.5 km at Ermitaño beach). This result raises the question as to the role of sandy beaches in the maintenance of parapatry and to their role as a reproductive isolation barrier. Ermitaño beach can be considered to be an obstacle that is difficult to overcome given the low dispersal capacity of spores and gametes in *L. nigrescens* species. However, many beaches are located between 18 and 42° S (810 stretches of sandy beach censused by Thiel et al. 2007, occurring every 1–4 km on average), some beaches stretch over 60 km (Concepción, 38° S) without any major associated genetic discontinuity (Tellier et al. 2009). Moreover, in the Aceituno-Choros contact zone, populations of the same species located to the north and south of the Aceituno (5 km) and Choros (17 km) beaches shared alleles at microsatellite loci and did not show any pronounced genetic differentiation (AMOVA results for the southern species), reflecting the permeability of these barriers to gene flow. Consequently, the presence of the sandy beach certainly decreases the

TABLE 3. Analyses of molecular variance on microsatellite data in the Aceituno-Choros contact zone.

Source of variation	Degree of freedom	Sum of squares	Variance component	Percentage of variation (%)	*P*-value
a. Sites grouped according to the species to which the individuals belong.					
Among species (northern and southern species)	1	153.235	0.61046	45.43	0.00267
Among sites within species	10	21.643	0.03562	2.65	<0.00001
Within sites	484	337.658	0.69764	51.92	<0.00001
Total	495	512.536	1.34372		
b. Only sites of the southern species, grouped according to their location with respect to Aceituno beach.					
Among groups (both sides of the beach)	1	7.017	0.04310	4.32	0.09990
Among sites within each side of the beach	4	5.854	0.01218	1.22	0.25564
Within sites	254	239.414	0.94257	94.46	0.00040
Total	259	252.285	0.99785		

probability of interspecific encounters (i.e., it acts as a prezygotic reproductive isolation mechanism), but it is unlikely to fully impede dispersal between populations of both species. It is highly likely that our estimation of intraspecific gene flow was limited by the low number of markers employed and particularly by the low polymorphism observed in the northern species. However, this pattern of spatially discrete species segregation suggests that other types of mechanisms act at very short distances to minimize effective dispersal across Ermitaño beach. Several nonmutually exclusive hypotheses can be invoked. First, differences in (micro-)habitat to the north and to the south of Ermitaño beach (as abiotic factor: temperature; or biotic factors: grazers or associated flora) may cause differential selection that limits establishment of the nonadapted species. Future studies are needed to identify such possible habitat differences at local scale and to determine if they affect only the distribution ranges of our model species or also other marine species. Second, there may be competitive exclusion between species. Third, if any incompatibility mechanism is acting between species, either complete or partial, the reproductive output of the migrant gametophytes would therefore be lower than that of the locals. This should be reflected by an intrinsic selection against the rare taxon (i.e., negative frequency-dependent selection), leading to a decrease in the effective dispersal between sites dominated by different species. In the case of our model species, the intrinsic selection against the locally rare taxon is probably of a major importance. Because of the limited dispersal capacity of spores, the ''migrant'' gametophytes are expected to be at a much lower frequency than the local gametophytes.

Theory predicts that the location of tension zones is ''attracted'' by dispersal barriers (Hewitt 1988, Goldberg and Lande 2007), meaning that its location will tend to be in places where a physical barrier to dispersal minimizes the chance of encounter between gametes of both species. In our study, dispersal barriers are beaches that may have attracted the tension zones created by one or several of the aforementioned mechanisms at the limits of each (micro)-habitats. The case of *L. nigrescens* species in the Aceituno-Choros region seems to fit this model of tension zones, but additional studies are needed to discriminate between genetic incompatibility and competitive exclusion mechanisms to explain this pattern.

Contrasting genetic diversity between southern and northern species. In the contact zone (29° S), genetic diversity of the southern species populations was greater than that of the northern species. This difference in genetic diversity seems to be a characteristic of this region. Accordingly, allelic richness (*A*) is higher at Pan de Azúcar (26° S, $A = 6.8$, Faugeron et al. 2009) than in the Choros region ($A = 2.7$, this study) for the northern species. Similarly, for the southern species, two of the four loci used in the present study (LESS2D1 and LESS2D22) are monomorphic at Las Cruces (33° S, Faugeron et al. 2009), whereas they are polymorphic in the Aceituno region (this study), with high allelic richness. Three nonexclusive hypotheses may explain this pattern. First, different histories between local populations could lead to differences in genetic diversity. The persistence of a southern species population with high genetic diversity and surrounded by northern species stands suggests that there may be remnants of a past distribution, whereas northern species populations may have experienced a more recent colonization, associated with an important founder effect. Second, the effect of recurrent contemporary gene flow from southern core populations to the northern range limit could be responsible for the high diversity observed in the Aceituno region. Despite the very limited dispersal capacity of the species and the important distance between these populations (60–200 km), this scenario is supported by the local oceanographic conditions, with the northward coastal current connecting the core population of the southern species to the Aceituno region (Thiel et al. 2007). In contrast, marginal populations of the northern species are probably less connected by currents to their core populations as they would require southward gene flow (i.e., countercurrent dispersal). Third, demographic processes and effective sizes of the local populations may differ among species either because of intrinsic characteristics of the species or because of environmental differences between the sites. However, as the spatial scale at which the species are segregating is much smaller than the ones considered in oceanographic studies, it is difficult to determine which environmental factor could promote such differences in demographic processes. The northern species seems to occupy sites characterized by non-upwelling coastal conditions in the transition zone (29° S–31° S, Tapia et al. 2009), whereas the southern species is generally found in well-known important upwelling centers within this particular region (Thiel et al. 2007, Tapia et al. 2009, Tellier et al. 2009). Because the disturbances induced by El Niño Southern Oscillations (ENSO) are strongly limited to the northernmost part of the Chilean coast (see for review Thiel et al. 2007), the populations from the overlapping region are not so massively affected as the northernmost populations (e.g., 20° S: Martínez et al. 2003). By consequence, ENSO events may have differential consequences on the two cryptic species of *L. nigrescens*, but this alone is unlikely to explain the pattern observed at higher latitudes (29° S–30° S) and at a small spatial scale (a few tens of kilometers). This is a promising clue to explain the differential distribution of the species not only at a wide scale (transition zone) but also at the small scale (contact zone). However,

environmental characteristics of populations need to be further studied, in parallel with comparative studies of the physiological responses of both species.

At a wider scale, the fragmentation of populations in the transition region, composed of a mosaic of sites occupied either by the northern or by the southern species, should in theory lead to genetic drift for populations at the limits of their species range (Bridle and Vines 2007, Eckert et al. 2008). Nevertheless, our results suggest that for the southern species, diversity is higher at the species northern range margin (Aceituno) than at the center of the species' range (Las Cruces, Faugeron et al. 2009). To confirm this result, additional central and marginal populations need to be analyzed. A more comprehensive comparative analysis of other contact zones located in this mosaic transition region would help resolve this question of genetic diversity with respect to species range. Local processes (those specific to a given contact zone) could then be distinguished from general processes that are associated with the ''contact'' of the two species (parapatry). In particular, this type of study would help distinguish between general reproductive isolation whatever the secondary contact zone and site-specific reinforcement processes, such as prezygotic barriers between hybridizing species, in response to selection against interspecific crosses (for review, see Servedio and Noor 2003).

Northern and southern species: two reproductively isolated species. The absence of any alleles shared between species, defined on the basis of mitochondrial lineages, suggests that there is no gene flow between these cryptic species in their contact zone. We did not detect any heterozygotes for species-diagnostic alleles, indicating an absence of hybrids. Furthermore, there was no discrepancy between mitochondrial and nuclear markers, suggesting that reproductive isolation between these species is complete and probably old. These results suggest that the two phylogenetic species identified by Tellier et al. (2009) fit the biological species concept (Mayr 1942). Because the species have contrasted distribution ranges along a latitudinal gradient, the study of their ecological niches is expected to reveal differences in tolerance to environmental factors varying latitudinally (e.g., UV radiation, desiccation, air and sea temperatures). Recently, Oppliger et al. (2011) presented one aspect of the ecological niches, evidencing a species-specific sex-ratio change under temperature stress.

The absence of interspecific crosses in this study can be partly attributed to the fact that these two species are not sympatric at local scale, limiting the possibility of hybridization between gametophytes. Indeed, hybridization in natural populations depends on both intrinsic (i.e., environment independent) and extrinsic reproductive barriers. Extrinsic barriers can greatly affect the success of interspecific crosses, either by favoring prezygotic barriers (phenology, synchrony of spawning, gamete incompatibility) or by limiting the success of any produced hybrids via habitat-dependent or negative frequency-dependent selection. Under controlled conditions, extrinsic barriers are not taken into account. Finally, hybrids may have limited fitness, with high mortality and/or limited fertility and thus may be present only in very low frequencies in natural populations. The complete life cycle of hybrid individuals obtained in the laboratory is only rarely studied, whereas in natural conditions, characterizing gene flow effectively integrates the fitness of hybrid individuals.

A particularly interesting extension of our study would be to directly measure interfertility by performing crosses under controlled conditions between the two *L. nigrescens* species to test whether there are intrinsic reproductive barriers, such as gamete incompatibilities or hybrid infertility. In addition, the recent development of hypervariable markers for kelp species (Billot et al. 1998, Whitmer 2002, Collens 2008, Dolman and Coleman 2008, Engel et al. 2008, Alberto et al. 2009, Faugeron et al. 2009) should provide the necessary tools for assessing the existence of reproductive barriers in both natural and laboratory conditions in a large number of kelp species and generalizing (or not) our results.

CONCLUSIONS

We detected a complete spatial segregation of these two cryptic kelp species. In addition, we demonstrated here a complete reproductive isolation in natural conditions. These findings open new questions regarding the role of genetic incompatibility and competitive exclusion mechanisms in maintaining kelp species segregation and species integrity. More generally, the availability of molecular tools should favor more studies on species delineation in kelps and other seaweeds for which cryptic diversity is increasingly being reported. In particular, comparisons between sympatric and parapatric species would contribute to better understanding the mechanisms that are at the origin of cryptic species formation and coexistence.

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Supplementary Material

The following supplementary material is available for this article:

Table S1. Values of the statistical estimator of pair-wise F_{ST} between sites and the associated *P*-values. Above the diagonal, *P*-values; below the diagonal, F_{ST} values. *P*-values lower than the set α threshold (0.05) after Bonferroni correction are shown in bold ($P < 0.00075$).

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